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PRINCIPAL INVESTIGATOR: Dr. John Meyers

CONTRACTING ORGANIZATION: University of Texas
Health Science Center
San Antonio, TX 78284-7758

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The human epidermal growth factor receptor (EGFR) promoter is activated by both wild-type and tumor-derived mutant p53. In this report we demonstrate that the EGFR promoter is activated by tumor-derived p53 mutants p53-143A, p53-175H, p53-248W, p53-275H and p53-281G. Using p53-281G as a model we show that the transactivation by mutant p53 requires different protein domains than wild-type p53 and does not require the wild-type p53-binding site. The minimal EGFR promoter from positions -104 to -20 which does not contain the wild-type p53-binding site is transactivated by the p53 mutants but not wild-type protein. Together the domain requirements and promoter sequence requirements indicate a difference in the mechanism of transactivation by wild-type and mutant p53. Transactivation of the EGFR promoter by mutant p53s may represent a mechanism by which tumor cells lose cell growth control.

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John H. Ivler-Meyer 10/12/96

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INTRODUCTION

p53 is a tumor suppressor that negatively regulates the cell cycle(11, 27, 28, 32, 34). Mutation in the p53 gene is a very common somatic genetic change found in human cancer(11, 17, 23, 27, 28, 32, 34,). Although the p53 gene is not required for normal development in mice, lack of p53 function confers an enormously elevated risk of developing cancer. p53 seems to regulate cell growth, at least in part, by transcriptional control. The level of wild-type p53 in normal cells is very low, but when cells are exposed to genotoxic simuli, p53 levels rise rapidly and initiate a program of cell death or stop the cell cycle at the G_1/S boundary, presumably for DNA repair. This "guardian-of-genome" (27)response of wild-type p53 is lost in many tumor cells because they have either inactivated their p53 genes by mutation or blocked the activity of p53 through the production of proteins that bind to it and neutralize it.

Wild-type p53 is a sequence-specific DNA-binding protein(11, 27, 28, 34). The DNA binding domain of wild-type p53 has been identified to be the central region of the protein covering conserved domains II to V(2, 7, 21,). Wild-type p53 also has a strong transcriptional activation domain (amino acids 1 to 42) (14, 35,). As expected, wild-type p53 is a sequence-specific transactivator for promoters with p53-binding sites(11, 27, 28, 34). Tumor-derived p53 mutants are in general defective in this sequence-specific transactivation. Several prospective target genes whose expression may be positively influenced by wild-type p53 have been identified. These include murine muscle creatine kinase, MDM2 (1,33), CIP1/WAF1/p21 (12), epidermal growth factor receptor (EGFR) (10), thrombospondin-1 (16), GADD45, Bax, human transforming growth factor alpha (36), insulinlike growth factor-binding protein 3 (3), human proliferating-cell nuclear antigen (PCNA) (2, 30, 37) and cyclin G (31).

Several groups have also demonstrated that overexpression of wild-type (but not mutant) human p53 inhibits the in vivo transcription of many cellular and viral promoters which lack p53-binding sites (9, 15, 39-41). p53-mediated transcriptional repression is released by adenovirus E1B p19 or cellular Bcl-2 proteins, suggesting that repression may be a part of the sequence of events in apoptosis (35). We and others have also reported that some cancer-derived p53 mutants activate the promoters of genes expressing PCNA, EGFR, multiple drug resistance MDR1, vascular endothelial growth factor, human interleukin-6, basic fibroblast growth factor and human heat shock protein 70 (HSP70) (6, 9, 42). This transactivation represents the only known biochemical "gain of function" for tumor-derived p53 mutants. Two critical hydrophobic amino acids (positions 22 and 23) in the N-terminal transactivation domain of mutant p53 are required for gain of function as judged by tumorigenesis assays and by transactivationof the MDR1 promoter suggesting that the transactivation ability of mutant p53 is required for gain of function.

Growth factors modulate cell growth and differentiation via interaction with their receptors. Epidermal growth factor (EGF) stimulates cell growth by directly activating its receptor (EGFR), leading to signal transduction through an intracellular cascade of second messengers (5, 13). EGFR is a transmembrane glycoprotein of 170 kDa with an extracellular EGF-binding domain (4, 5, 13). EGFR acquires oncogenic properties when its structure or regulation becomes disrupted, as was found in transformed cells, tumors, and established cell lines (13, 36, 38,). Thus, regulation of EGFR transcription should be

critical for normal cell growth and proliferation. Earlier, we demonstrated that human p53 activates the EGFR promoter in a dose-dependent manner in vivo (10). This suggests that the p53 growth regulatory pathway may intersect the pathway used by growth factors to regulate cell proliferation. We have now shown that transforming mutants of p53 also activate the EGFR promoter. This observation may signify a pathway by which mutant p53 may induce cell proliferation.

Three promoters, EGFR (10), MDR1 (6, 16,), and PCNA (9, 16,) are transactivated by both wild-type and mutant p53. In last year's annual report we described the identification and characterization of a wild-type p53 DNA binding sequence in the EGFR promoter. This DNA sequence was shown to be required for wild-type mediated transactivation of the EGFR promoter. That data will be published in the November 1996 issue of Molecular and Cellular Biology. In the present study we have examined EGFR promoter transactivation by tumor-derived p53 mutants. We show that activation of the EGFR promoter by mutant p53 requires promoter sequences previously identified as the basal promoter (26). This promoter element does not contain the wild-type p53 response element indicating different mechanisms of transactivation between wild-type and mutant p53. Analysis of the p53 protein domains requried for activation of the EGFR revealed that domain requirements also differ between wild-type and mutant p53.

MATERIALS AND METHODS

DNA plasmids. The p53 expression plasmids contain a wild-type or mutant p53 cDNA under the regulation of the human cytomegalovirus immediate-early promoter in the pCMV-Bam expression vector (22, 39). The series of N- and C-terminal deletion derivatives were generated as described previously (41).

The chloramphenicol acetyl transferase (CAT) plasmids utilized the *Escherichia coli* CAT gene under the transcriptional control of human EGFR promoter sequences. The 3' end of each of the following EGFR-CAT constructs (26, a gift from Glenn T. Merlino) is at -20 relative to the EGFR translation start site, while the 5' ends map to the following restriction sites and positions: pERCAT-1 (Hind III, -1109); pERCAT-9 (Ava II, -389) and pERCAT-15 (Ava I, -104). pER-1.CAT is a plasmid with the EGFR upstream sequences from -1109 to -20 cloned upstream of the CAT gene in the pCATbasic vector (Promega) (10).

Cell culture and transfection. Human osteosarcoma (Saos-2) cells were cultured and transfected by the calcium phosphate-DNA coprecipitation method, as described previously (39). In a typical experiment, 1-2 X 10^6 cells were cotransfected with 2.5 ug of a CAT construct and 5 ug of a p53 expression plasmid (or 5 ug of the expression vector without p53 sequences as a negative control). All transfection experiments were repeated multiple times (three times or more).

CAT assay. Cells were harvested 36 to 40 h posttransfection and lysed by three successive cycles of freezing and thawing. Extracts were normalized for protein concentration and assayed for CAT activity (39). Because mutant p53s activate different promoters to different extents (9, 10, 15, 39), it was not possible for us to use an internal control such as pSVβGal or RSVβGal. A similar situation has been recognized for simian virus 40 T antigen-mediated regulation of promoters(18). Therefore, we have done multiple independent experiments with different DNA preparations and calculated the standard deviations. CAT activity was detected by thin-layer chromatographic seperation of [^{14}C]chloramphenicol from its acetylated derivatives followed by autoradiography. Quantitation was done with a Phosphoimager (Molecular Dynamics).

RESULTS

Wild-type human p53 and its tumor-derived mutants transactivate the human EGFR promoter. To compare the transactivation efficiencies of wild-type and tumor-derived p53, we performed transient-transfection assays with a promoter-CAT construct containing the entire EGFR promoter (pER-1.CAT) (10). We transfected Saos-2 cells with pER-1.CAT and wild-type p53 or mutant p53 expression plasmids (or expression vector alone). Figure 1 shows a representative CAT assay result. p53 levels were analyzed by Western blot (immunoblot) analysis of the protein extracts used for the CAT assays. Even taking into account the variation of the level of p53 expressed, wild-type p53 remains the weakest and p53-281G is the strongest transactivator (data not shown). Thus, p53 mutants differ in their transactivation efficiency from each other and from the wild-type protein.

Protein domains required for mutant p53-mediated transcriptional activation of the human EGFR promoter. In the previous annual report the amino terminus of wild-type p53 was shown to be required for activation of the EGFR promoter while the carboxy terminal 66 amino acids are dispensable. Further deletion of the C-terminus eliminated wild-type transactivation. To determine the domain requirements of mutant p53 for transactivation of the EGFR promoter a similar set of amino- and carboxy-terminal deletions were made in the mutant p53 protein. Saos-2 cells were cotransfected with pER-1.CAT (containing the EGFR promoter from -1109 to -20) (10) and with a plamid expressing either the mutant p53-281G protein or one ot its N- or C-terminal deletion derivatives (or with the pCMV-Bam expression vector). The results in Fig. 2B indicate requirements for the N-terminal transactivation domain and the C-terminal region involved in oligomerization and non-sequence-specific DNA binding. In Fig. 2A the domain requirements for wild-type p53 transactivation of the EGFR promoter are given (previously presented in the October 1995 annual report) to allow comparison. The C-terminal domain requirements differ for transactivation of the EGFR promoter by wild-type and mutant p53 (compare del 393-327). This indicates a possible difference in mechanism of transactivation used by wild-type and mutant p53.

Activation of the minimal EGFR promoter by p53-281G. To determine the sequence requirements for transactivation by p53-281G, we used a set of EGFR promoter deletion mutants (Fig 3A) (kind gifts from G. Merlino). We performed cotransfection experiments in Saos-2 cells with different promoter deletion mutant-CAT constructs and the p53-281G expression plasmid (or expression vector alone). Figure 3B depicts a representativeCAT assay result. All the deletion mutants, including the one with a deletion up to -104 (ER-1 ΔAvaI) were activated significantly by p53-281G (Fig. 3B, compare lanes 1 and 2, lanes 3 and 4, and lanes 5 and 6). The EGFR upstream sequences up to -104 define the minimal promoter of EGFR (26). These results suggest that p53-281G activates the minimal EGFR promoter possibly by interacting with the machinery required for basal transcription. This observation is similar to that observed for the PCNA, human immunodificiency virus long terminal repeat, and human HSP70 promoters (9, 10, 40, 42,). It is significant that wild-type p53 does not transactivate the minimal EGFR promoter without the p53-binding site (October 1995 annual report,) showing that

the mechanisms of transactivation of the EGFR promoter by wild-type and mutant p53 are different.

DISCUSSION

In this report we present evidence that tumor-derived mutants of p53 transactivate the EGFR promoter, a promoter we previously observed to contain a wild-type p53 transcriptional response element (October 1995 annual report,). We show that the p53-binding site is not required for transactivation by the mutant proteins (Fig. 3) consistent with the previous demonstration that baculovirus purified p53-281G does not bind to the this site (October 1995 annual report,). The minimal promoter defined by the AvaI deletion mutant of the EGFR promoter is activated efficiently by tumor-derived p53 mutants. Mutant p53-mediated transactivation of the EGFR promoter requires the oligomerization domain, whereas wild-type p53-mediated transactivation does not (Fig. 2). These results suggest that mutant proteins use a different mechanism to transactivate the EGFR promoter. Since many tumor-derived p53 mutants are defective in sequence-specific DNA binding (11, 27, 28, 34), it is possible that mutant p53 activates the EGFR promoter after being recruited on the DNA by some other transcription factor. Since the EGFR minimal promoter is activated by mutant p53, it could serve as a simple model to understand mutant p53-mediated transactivation.

The minimal EGFR promoter has one Sp1-binding site, and transcription from the minimal promoter is dependent on Sp1 (43). Since Sp1-mediated transcriptional activation may involve $TAF_{II}110$ and $TAF_{II}250$ (24), involvement of the $Sp1/TAF_{II}110/TAF_{II}250$ system in mutant p53-mediated transactivation may be speculated. Involvement of Sp1 in p53-mediated transactivation has been noted previously for transactivation of HIV LTR (19, 20). The EGFR minimal promoter also contains a thyroid hormone receptor binding site which overlaps the Sp1 binding site . This element has been shown to inhibit Sp1 dependent transcription in the presence of ligand activated thyroid hormone receptor and retinoic acid receptors(43). Interestingly, it has been observed that p53 binds to thyroid hormone receptors in vitro and some tumor-derived mutant p53s increase human thyroid receptor β1 dependent transcription (44). The AvaI deletion mutant of the EGFR promoter also contains element(s) that confer responsiveness to phorbol 12-myristate 13-acetate (PMA). This responsiveness was due, in part, to a functional activator protein 2 (AP-2) DNA binding site. A consensus binding site for activator protein 1 (AP-1) was identified, by DNA sequence comparison, within this same EGFR promoter fragment and may contribute to the PMA responsiveness (25). Thus, the EGFR promoter AvaI deletion is an important regulatory element that contains multiple transcription factor binding sites and is also the minimal sequence required for tumor-derived mutant p53-mediated transcriptional activation.

Our lab has previously shown that mutant p53 activates transcription from synthetic Sp1, NF-κB and ATF binding sites (40). This has lead to the hypothesis that tumor-derived mutant p53 may act as a co-activator of transcription. This function would "bridge" transcription factors to the basal transcription machinery necessitating transcription factor binding sites for mutant p53 transcriptional activation. Deletion and point mutations of the EGFR AvaI deletion fragment are being made to identify transcription factor binding sites, and thus the transcription factor(s) required for mutant p53-mediated transcriptional activation. The identified transcription factor(s) will be analyzed for the ability to interact with mutant and wild-type p53 proteins. Also, studies

are under way to determine if mutant p53 interacts with proteins associated with the basal transcription apparatus (such as the TATA-binding protein, $TF_{II}B$ and $TAF_{II}250$). Both activities are consistent with the function of a transcriptional co-activator.

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FIGURES

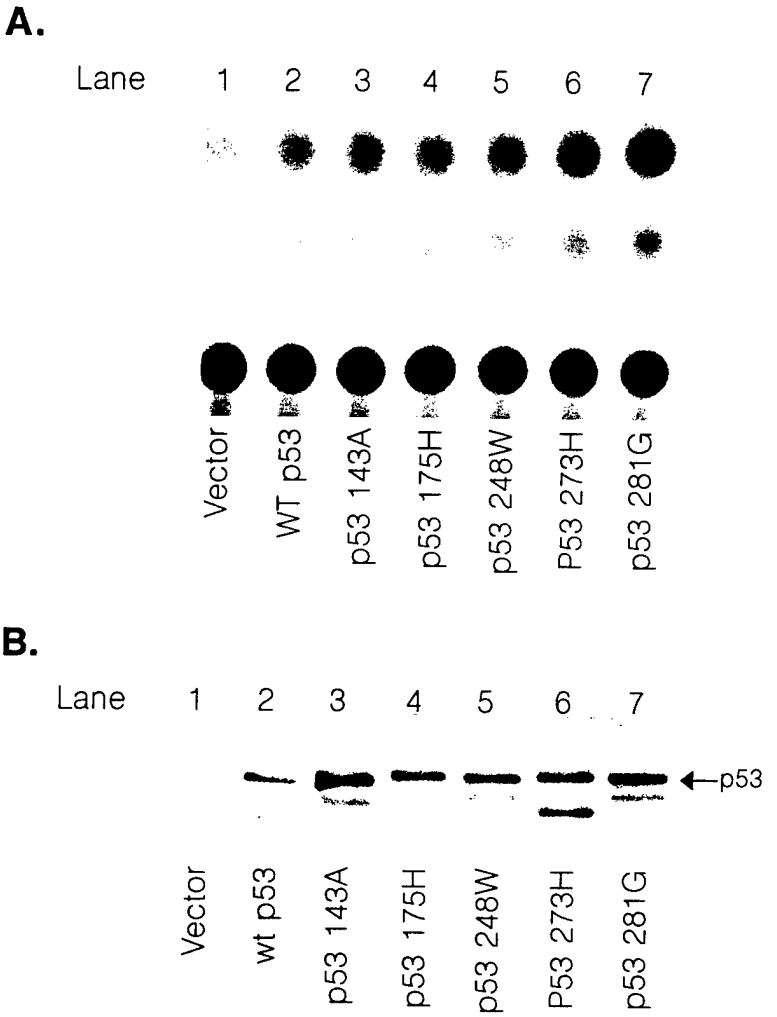

FIG. 1. Effect of expression of wild-type p53 and its tumor-derived mutants on EGFR promoter function in Saos-2 cells. Saos-2 cells were transfected with 2.5 ug of EGFR.CAT (pER-1.CAT) containing EGFR promoter sequences from -1109 to -20 upstream of the bacterial CAT gene and 5 ug of vector alone or 5 ug of an expression plasmid for wild-type (WT) p53 or one of its mutants (p53-143A, p53-175H, p53-248W, p53-273H or p53-281G). After transfection, cells were treated as described in Material and Methods. (A) Representative CAT assay result. The average fold transactivations by wild-type and mutant p53 are as follows: wild-type p53, 3.03 ± 0.55; p53-143A, 4.15 ± 2.15; p53-175H, 4.62 ± 1.01; p53-248W, 5.12 ± 0.75; p53-273H, 12.4 ±5.05; p53-281G, 15.2 ± 3.36. (B) Western blot analysis carried out with equal amounts of protein from each CAT assay extract, using a monoclonal antibody (Pab1801 [Ab2]; Oncogene Science). The position of the p53 band is shown by an arrow

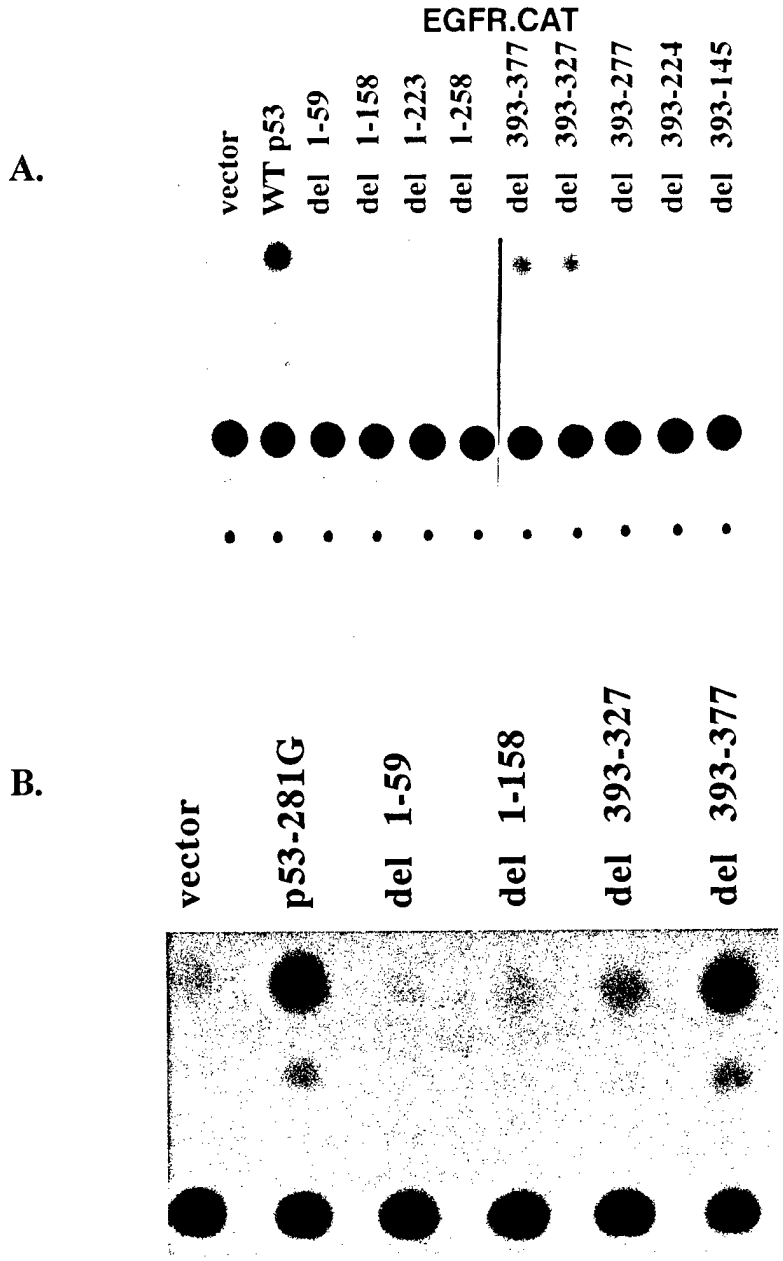

FIG. 2. Protein domain requirements for transcriptional activation of the human EGFR promoter by wild-type (WT) p53 (A) and its mutant derivative p53-281G (B). To determine the domains on the wild-type and mutant protein required for p53-mediated transactivation of the human EGFR promoter, a series of N- and C-terminal deletion derivatives was used. Saos-2 cells were cotransfected with 2.5 ug of pER-1.CAT (containing the EGFR promoter from -1109 to -20) along with 5 ug of a plasmid expressing either the intact p53 protein or one of its N- or C-terminal deletion derivatives (or with 5 ug of the pCMV-Bam expression vector).

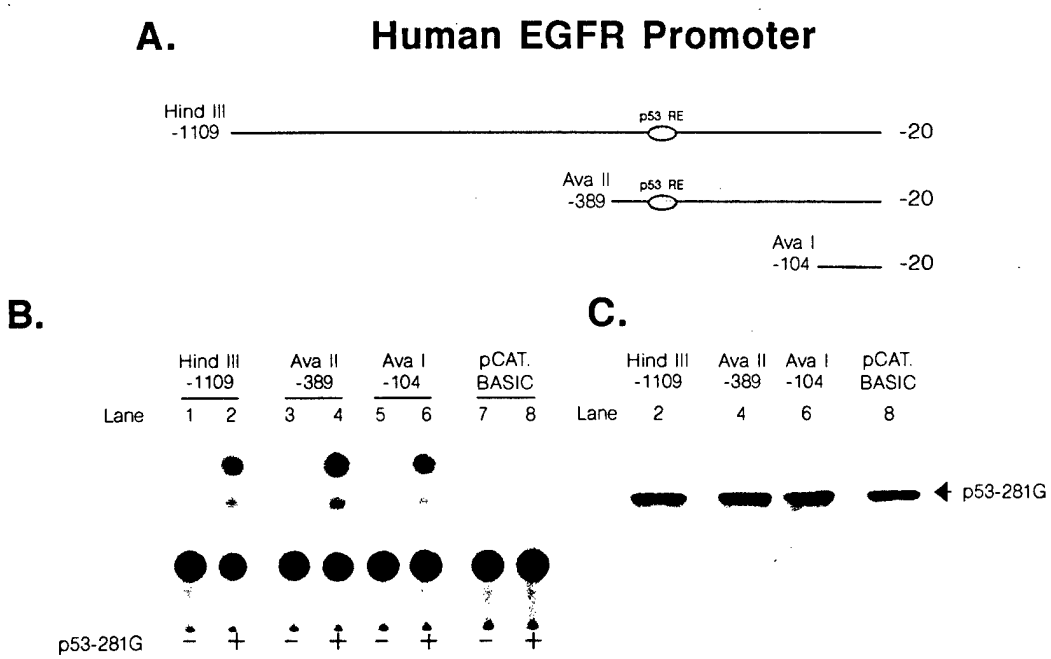

FIG. 3. Activation of the minimal EGFR promoter by p53-281G. Saos-2 cells were transfected with 5 ug of pCMV p53-281G (or expression vector alone without insert) and 2.5 ug of pER-1.CAT or one of the promoter deletion mutants shown at the top. (A) EGFR promoter deletion mutants used in the assays. The p53-binding site is indicated. (B) CAT assays were performed as described in Materials and Methods. The average fold transactivations of different promoter deletion mutants by wild-type human p53 are as follows: pER-1.CAT (promoter extending up to HindIII at -1109), 26 ± 18; pER-1.CAT del AvaII, 33.6 ± 26; pER-1.CAT del AvaI, 16.5 ± 5.9. (C) Western blot analysis carried out with equal amounts of protein from each CAT assay extract, using a monoclonal anti-p53 antibody (Pab1801 [Ab2]; Oncogene Science).

BIBLIOGRAPHY

John H. Ludes-Meyers Ph. D. is the sole personnel receiving pay from the negotiated effort.

Below are abstracts from one publication and one scientific meeting resulting from the funds provided by this grant:

Transcriptional Activation of the Human Epidermal Growth Factor Receptor Promoter by Human p53

JOHN H. LUDES-MEYERS, MARK A. SUBLER, CHITTARI V. SHIVAKUMAR, RUBEN M. MUNOZ, PENG JIANG, JOHN E. BIGGER, DORIS R BROWN, SWATI PALIT DEB, AND SUMITRA DEB

The human epidermal growth factor receptor (EGFR) promoter is activated by both wild-type and tumor-derived mutant p53. In this communication, we demonstrate that EGFR promoter sequence requirements for transactivation by wild-type and mutant p53 are different. Transient-expression assays with EGFR promoter deletions identified a wild-type human p53 response element, 5'-AGCTAGACGTCCGGGCAGCCCCCGGCG-3', from positions -265 to -239. Electrophoretic mobility shift analysis and DNase I footprinting assays indicated that wild-type p53 binds sequence specifically to the response element. Using circularly permuted DNA fragments containing the p53-binding site, we show that wild-type p53 binding induces DNA bending at this site. We further show that the EGFR promoter is also activated by tumor-derived mutants p53-143A, p53-175H, p53-248W, p53-273H, p53-281G. However, the transactivation by mutant p53 does not require the wild-type p53 -binding site. The minimal EGFR promoter from positions -104 to -20 which does not contain the wild-type p53-binding site is transactivated by the p53 mutants but not the wild-type protein, showing a difference in the mechanism of transactivation by wild-type and mutant p53. Transactivation of the EGFR promoter by p53 may represent a novel mechanism of cell growth regulation.

Cold Spring Harbor Laboratory

Cancer Genetics & Tumor Suppressor Genes (Oral)

John H. Ludes-Meyers

DIFFERENTIAL INVOLVEMENT OF $TAF_{II}250$ IN p53-MEDIATED REGULATION OF THE HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR PROMOTER. John H. Ludes-Meyers, Mark A. Subler, Chittari V. Shivakumar, Doris R. Brown, Swati Palit Deb, and Sumitra Deb, Department of Microbiology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284

While p53 is an intracellular regulator of cell proliferation, epidermal growth factor modulates cell growth by binding to the extracellular domain of its receptor (EGFR). We demonstrate that the human EGFR gene promoter is transcriptionally activated by both wild-type p53 and tumor derived mutants. We performed a structure-function analysis of wild-type p53 and p53-281G. *In vivo* transcription analyses indicate that the N-terminal acidic activation domain as well as the oligomerization domain is necessary for successful transactivation by wild-type and mutant p53. A high affinity binding site for wild-type p53 within the promoter (-265 to -239) is located just downstream from the major transcriptional start site. Binding to this site wild-type p53 also bends DNA. Using cells devoid of endogenous p53 and *in vivo* transcriptional analysis we show that this binding site is necessary for transcriptional activation by wild-type but not by mutants indicating a difference in their mechanism of action.

The sequence from -105 to -20, previously defined as the basal enhancer/promoter, is sufficient for mutant p53 transcriptional activation. This element contains overlapping binding sites for Sp1 and the Thyroid hormone receptor. *In vivo* binding assays indicate an interaction of Sp1 and p53-281G. Since Sp1-mediated transactivation involves $TAF_{II}250$, we investigated the role of $TAF_{II}250$ in mutant p53-mediated transactivation of the EGFR promoter. *In vivo* transcriptional analyses using BHK-C21-derived ts13 cells with temperature sensitive $TAF_{II}250$ show no transactivation of the EGFR promoter by p53-281G at the nonpermissive (39°C) temperature although significant transactivation can be seen at permissive temperature (30°C). Transactivation at 39°C can be rescued by exogenous $TAF_{II}250$. Transactivation of a synthetic promoter construct containing an ATF-binding site and a TATA box also needed active $TAF_{II}250$. However, wild-type p53 transactivated another synthetic promoter with four p53-binding sites and a TATA element at 39°C indicating that $TAF_{II}250$ is not absolutely essential for transactivation by wild-type p53. Our data thus suggest wild-type and mutant p53 differ in the mechanism of transactivation; mutant p53 may act as a coactivator, and perhaps transactivates the EGFR promoter by interacting with Sp1 requiring $TAF_{II}250$ for its function.